THIS PAGE BLANK (USPTO)

REC'D 2 3 NOV 1999

WIPO   PCT

**Patent Office
Canberra**

I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND
SALES hereby certify that annexed is a true copy of the Provisional specification
in connection with Application No. PP 6274 for a patent by THE UNIVERSITY
OF QUEENSLAND filed on 02 October 1998.
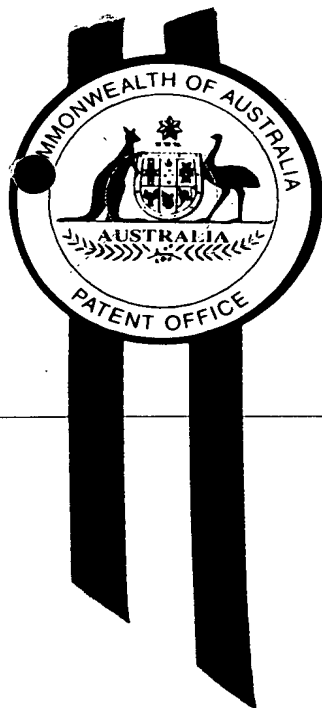
WITNESS my hand this
Eighteenth day of November 1999

LEANNE MYNOTT
TEAM LEADER EXAMINATION
SUPPORT AND SALES

The University of Queensland

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Novel Peptides - I"

The invention is described in the following statement:

NOVEL PEPTIDES - I

The present invention relates to novel peptides and derivatives thereof useful as inhibitors of neuronal amine transporters of neurotransmitters such as noradrenaline, seratonin, dopamine,

5 glutamic acid and glycine. The invention also relates to pharmaceutical compositions comprising these peptides, nucleic acid probes useful in finding active analogues of these peptides, assays for finding compounds having neuronal noradrenaline transporter inhibitory activity and the use of these peptides in the prophylaxis or treatment of condistions such as but not limited to incontinence, cardiovascular conditions and mood disorders.

10

The marine snails of the genus *Conus* (cone snails) use a sophisticated biochemical strategy to capture their prey. As predators of either fish, worms or other molluscs, the cone snails inject their prey with venom containing a cocktail of small bioactive peptides. These toxin molecules, which are referred to as conotoxins, interfere with neurotransmission by targeting a variety of

15 receptors and ion-channels. The venom from any single *Conus* species may contain more than 100 different peptides. The conotoxins are divided into classes on the basis of their physiological targets. To date, ten classes have been described. The ω-conotoxin class of peptides target and block voltage-sensitive Ca^{2+}-channels inhibiting neurotransmitter release. The α-conotoxins and ψ-conotoxins target and block nicotinic ACh receptors, causing

20 ganglionic and neuromuscular blockade. Peptides of the μ-conotoxin class act to block voltage-sensitive Na^+-channels inhibiting muscle and nerve action potentials. The δ-conotoxins target and delay the inactivation of voltage-sensitive Na^+-channels, enhancing neuronal excitability. The κ-conotoxin class of peptides target and block voltage-sensitive K^+-channels, and these also cause enhanced neuronal excitability. The conopressins are vasopressin receptor antagonists

25 and the conantokins are NMDA receptor antagonists. Recently, the prototype of a new γ-conotoxin class, which targets a voltage-sensitive nonspecific cation channel, and of a new σ-conotoxin class, which antagonises the $5HT_3$ receptor, have been described.

It has now been found that a new class of conotoxin exists, hereinafter referred to as the χ-

conotoxin class, which are characterised by having the ability to inhibit neuronal amine transporters.

Compounds which inhibit neurotransmitter reuptake have been found to be useful in the
5 treatment of lower urinary tract disorders, such as urinary incontinence, detrusor instability and interstitial cystitis. One such compound is "imipramine" which, in addition to inhibiting noradrenaline reuptake, has been shown to affect calcium channel blockade, and to exhibit anticholinergic, local anaesthetic activity and a number of other effects. Other compounds capable of inhibiting noradrenaline reuptake are described in U.S. Patent 5,441,985. These
10 compounds are said to have a reduced anticholinergic effect relative to imipramine.

In the case of the peptides of the present invention this inhibition of neurotransmitter reuptake is achieved by selectively inhibiting the neuronal neurotransmitter transporter, such as the noradrenaline transporter, which functions to rapidly clear released noradrenaline from the
15 synapse back into neurons.

The peptides of the present invention are the first peptides to have activity in inhibiting an amine transporter. All other conotoxin peptides characterised to date target ion channels or receptors on cell surfaces.
20
According to one aspect of the present invention there is provided an isolated, synthetic or recombinant χ-conotoxin peptide having the ability to inhibit a neuronal amine transporter.

Preferably, the neuronal amine transporter is the neuronal noradrenaline transporter.
25
The χ-conotoxin peptide may be a naturally occurring peptide isolated from a cone snail, or a derivative thereof.

Preferably the χ-conotoxin peptide is χ-MrIA or χ-MrIB, or a derivative thereof. χ-MrIA and χ-MrIB may be isolated from the venom of the mollusc hunting cone snail, *Conus marmoreus*. They are both peptides of 13 amino acid residues in length, and contain 2-disulphide bonds; the peptides show most homology to members in the α-conotoxin class, which act as nicotinic ACh

5 receptor antagonists.

The amino acid sequences of χ-MrIA and χ-MrIB are as follows:

    χ-MrIA   NGVCCGYKLCHOC   SEQ ID NO. 1

10

    χ-MrIB   VGVCCGYKLCHOC   SEQ ID NO. 2

The C-terminus may be a free acid or amidated.

15 In the sequences above the "O" refers to 4-hydroxy proline (Hyp). This amino acid residue results from post translational modification of the encoded peptide and is not directly recoded by the nucleotide sequence.

Preferably, the χ-conotoxin peptide is a selective inhibitor of the neuronal noradrenaline

20 transporter. The terms "selective" and "selectively" as used herein mean that the activity of the peptide as an inhibitor of neuronal noradrenaline transporter is considerably greater than any activity at the α_1-adrenoceptors.

U.S. Patent 5,441,985 indicates that inhibitors of noradrenaline reuptake which have a

25 negligible anticholinergic effect are particularly useful in the treatment of lower urinary tract disorders. It has been found that χ-MrIA also has no detectable anticholinergic effect.

Accordingly in a preferred embodiment of the invention there is provided an isolated, synthetic

- 4 -

or recombinant χ-conotoxin peptide having the ability to selectively inhibit neuronal noradrenaline transporter, and having negligible or no anticholinergic effect.

χ-MrIA has also been found to have no activity as a sodium channel blocker or as an inhibitor
5 of dopamine transporter. The absence in χ-MrIA, of these additional pharmacological activities commonly associated with other noradrenaline transporter inhibitors and in preferred peptides according to the invention, makes these peptides useful pharmacological tools.

The χ-conotoxin peptides according to the invention may be naturally occurring peptides, such
10 as χ-MrIA and χ-MrIB, or may be derivatives of naturally occurring peptides.

The term "derivative" as used herein in connection with naturally occurring χ-conotoxin peptides, such as χ-MrIA and χ-MrIB, refers to a peptide which differs from the naturally occurring peptides by one or more amino acid deletions, additions, substitutions, or side-chain
15 modifications. Such derivatives which do not have the ability to inhibit neuronal noradrenaline transporter do not fall within the scope of the present invention.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may
20 be classified as "conservative", in which case an amino acid residue contained in a polypeptide is replaced with another naturally-occurring amino acid of similar character either in relation to polarity, side chain functionality or size, for example Ser↔Thr↔Pro↔Hyp↔Gly↔Ala, Val↔Ile↔Leu, His↔Lys↔Arg, Asn↔Gln↔Asp↔Glu or Phe↔Trp↔Tyr. It is to be understood that some non-conventional amino acids may also be suitable replacements for the
25 naturally occurring amino acids. For example ornithine, homoarginine and dimethyllysine are related to His, Arg and Lys.

Substitutions encompassed by the present invention may also be "non-conservative", in which

an amino acid residue which is present in a peptide is substituted with an amino acid having different properties, such as naturally-occurring amino acid from a different group (eg. substituting a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

5

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Preferably, amino acid substitutions are conservative.

10

Additions encompass the addition of one or more naturally occurring or non-conventional amino acid residues. Deletion encompasses the deletion of one or more amino acid residues.

As stated above the present invention includes peptides in which one or more of the amino acids
15 has undergone sidechain modifications. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups
20 with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

25

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid

or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation

5 with cyanate at alkaline pH. Any modification of cysteine residues must not affect the ability of the peptide to form the necessary disulphide bonds. It is also possible to replace the sulphydryl groups of cysteine with selenium equivalents such that the peptide forms a diselenium bond in place of one or more of the disulphide bonds.

10 Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

15 Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Proline residue may be modified by, for example, hydroxylation in the 4-position.

20 A list of some amino acids having modified side chains and other unnatural amino acids is shown in Table 1.

25

- 7 -

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug

D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
5 D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
10 D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
15 D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
20 D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
25 D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg

D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
5 D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
10 N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
15 γ-aminobutyric acid	Gabu	N-(p-hydroxyphenyl)glycine	Nhtyr
L-t-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
20 L-α-methylaspartate	Masp	L-α-methyl-t-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
25 L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr

L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethylglycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethylglycine	Nnbhe
5 1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc	O-methyl-L-serine	L-Ser(Me)
		O-methyl-L-homoserine	L-HSer(Me)

These types of modifications may be important to stabilise the peptide if administered to an
10 individual or used as a diagnostic reagent.

Other derivatives contemplated by the present invention include a range of glycosylation variants from a completely unglycosylated molecule to a modified glycosylated molecule. Altered glycosylation patterns may result from expression of recombinant molecules in different
15 host cells.

The χ-conotoxins of the present invention are typically amidated at the C-terminal, however compounds with a free carboxyl terminus or other modifications at the C-terminal are considered to be within the scope of the present invention. Preferably the peptides are amidated
20 or have a free carboxyl at the C-terminal.

Preferably the derivatives of naturally occurring χ-conotoxin peptides will retain the Cys residues and characteristic disulphide bonding pattern. Derivatives may include additional Cys residues provided they are protected during formation of the disulphide bonds.

25

In modification to form derivatives of naturally occurring χ-conotoxin peptides it is useful to compare the amino acid sequences of active naturally occurring peptides to determine which, if any, of the residues are conserved between active species. Substitution of these conserved residues, while not prohibited, is less favoured than substitutions of non-conserved residues.

Derivatives where Ala replaces one or more residues can be used to identify the pharmacophore. Preferably only one or two amino acids is replaced with Ala at a time. Additional new peptides can be made where charged, polar or hydrophobic residues, respectively, are replaced to assist defining more precisely the type of interactions involved in the binding of this pharmacological

5 class of peptide to its receptor. Non-conservative replacements, where charge is reversed, or polar residues replace hydrophobic residues, can further identify residues involved in binding. All of these peptides have potential to show improved potency, or greater selectivity. Non-native amino acid changes could also be included to improve potency, selectivity and/or stability.

10

Exposed residues are most likely to be involved in receptor binding and can be systematically replaced. Particular emphasis is placed on changing residues involved in binding and residues just on the periphery of the pharmacophore, using longer side chain forms or non-conserved changes to pick up additional binding interactions for improved potency and/or selectivity.

15 Reducing or enlarging loop sizes and the tail of MrIA or MrIB further modifies activity.

It is noted that MrIA and MrIB are composed of a tail (residues 1-3), and two loops (residues 6-9 and 11-12), however the χ-conotoxin peptides and derivatives of the present invention are not restricted to those having this particular arrangement of amino acids and disulphide bonds.

20 Other arrangements are also possible, and provided the resultant peptide has the requisite activity, a peptide will fall within the scope of the present invention. Preferably the peptides will have at least two cysteine residues and at lease one disulphide bond, or more preferably four cysteine residues and two disulphide bonds.

25 The connectivity of the disulfide bonds in these peptides may be A-B/C-D, A-C/B-D or A-D/B-C, the latter being preferred for MrIA and MrIB. A, B, C and D refer to the first, second, third and fourth Cys residues involved in disulphide bond formation, respectively.

- 12 -

These peptides can also be labelled and used to establish binding assays to identify new molecules that act at the same site. For example, labelled ligand of MrIA or MrIB could have tritium included or may have radio-active iodine or similar attached through a Tyr or other appropriate residue. A Tyr scan through each peptide will establish a suitable location for
5 incorporation of the Tyr. The inhibition of binding of such labelled peptides to tissue homogenates or expressed adrenoceptors by compounds or mixtures would permit identification of new peptides active at this site, including peptides present in serum and nerve and muscle tissue of mammals, including human tissues. The assay will also allow identification of non-peptide molecules that also act at the same site as MrIA and MrIB, and that may have utility as
10 orally active forms of these peptides. Labelled peptides will additionally permit autoradiographic studies to identify the location of the peptide binding across various tissues.

Portions of these sequences can be used to search ESTR data bases to identify in mammals peptides or proteins that contain related sequence information that could be used to identify
15 endogenous ligands that act in a similar manner in mammals.

Chimeras of χ-conotoxins such as MrIA, with other conotoxins or additionally with other peptides or proteins, can be made to engineer the activity into other molecules, in some instances to produce a new molecule with extra functionality. This would preferably be done
20 using the segment or segments of the sequence of these peptides that contain the pharmacophore. Where the pharmacophore is discontinuous, the segments making up the pharmacophore should be positioned in the new construct to allow binding to the receptor. Chimeras with other conotoxins may include additional Cys residues and additional disulphide bonds.
25

It is common for conotoxin peptides within an activity class to have a similar pattern of disulphide bonding, with peptide loops between the respective cysteine residues. For χ-MrIA and χ-MrIB disulphide bonds link the first and fourth, and the second and third cysteine

residues. This pattern is different from the binding pattern observed for α-conotoxin peptides. Despite this difference chimeric derivatives may be prepared by substituting a loop of a χ-conotoxin peptide with the loop comprising a sequence from another peptide, including α-conotoxin.

5

The invention also includes dimers, trimers, etc. of χ-conotoxin peptides as well as χ-conotoxin peptides bound to other peptides.

Preferably the χ-conotoxin peptides according to the invention have 10 to 30 amino acids, more 10 preferably 11 to 20.

The complete gene sequence for the naturally occurring χ-conotoxin peptides may be obtained using a combined 5' RACE and 3' RACE strategy coupled with cloning and DNA sequencing.

15 The χ-conotoxin peptides according to the present invention are active in inhibiting neuronal noradrenaline transporter. Such activity in pharmacological agents is associated with activity in the prophylaxis or treatment of diseases or conditions of the urinary or cardiovascular systems.

20 Accordingly the present invention provides a method for the treatment or prophylaxis of urinary or cardiovascular conditions or diseases or mood disorders including the step of administering to a mammal an effective amount of an isolated, synthetic or recombinant χ-conotoxin peptide having the ability to inhibit neuronal noradrenaline transporter.

25 Examples of diseases or conditions of the incontinence system include urinary and fecal incontinence. Examples of cardiovascular diseases or conditions include arrhythmias of various origins and coronary heart failure. Examples of mood disorders include depression, anxiety and cravings, such as smoking.

Preferably the mammal is in need of such treatment although the peptide may be administered in a prophylactic sense.

The invention also provides a composition comprising an isolated, synthetic or recombinant -

5   conotoxin peptide having the ability to inhibit neuronal noradrenaline transporter, and a pharmaceutically acceptable carrier or diluent.

Preferably the composition is in the form of a pharmaceutical composition.

10 There is also provided the use of an isolated, synthetic or recombinant χ-conotoxin peptide having the ability to inhibit neuronal noradrenaline transporter in the manufacture of a medicament for the treatment or prophylaxis of urinary or cardiovascular conditions or diseases, or mood disorders.

15 The invention will now be described with reference to the accompanying drawings and examples, however it is to be understood that the particularity of the following description is not to supersede the generality of the preceding description of the invention.

Referring to the figures:

20

Figure 1 is a graphical representation showing the typical effect of χ-MrIA on the time course of the contraction of the bisected rat prostatic vas deferens to field stimulation with a single supramaximal pulse (55 V, 1 ms). χ-MrIA (30 nM-3μM) was added to the organ bath cumulatively in half log unit steps.

25

Figure 2 is a graphical representation of the effect of χ-MrIA, -MrIA on contractile responses of bisected portions of the rat epididymal vas deferens to exogenous α_1-adrenoceptor agonists. (a) log concentration-response curves to noradrenaline in the absence (O) and the

- 15 -

presence of 1μM ($\triangle$) or 3 μM ($\square$) in χ-MrIA, -MrIA. (b) log concentration-response curves to methoxamine in the absence ($\bigcirc$) and the presence ($\square$) of 3 μM χ-MrIA. Each data point in (a) and (b) represents the mean $\pm$SEM of observations from 4-5 individual tissue preparations. Some error bars are obscured by the symbols.

5

Figure 3 is a graphical representation showing inhibition by χ-MrIA on the desipramine sensitive accumulation of [^{3}H]-noradrenaline by Chinese Hamster Ovary (CHO) cells transfected with the cDNA clone for the human neuronal noradrenaline transporter. Accumulation of [^{3}H]-noradrenaline is expression as a percentage of the cellular uptake in the

10 absence of χ-MrIA. Data points represent the mean $\pm$SEM or observations from 4 separate experiments.

Figure 4 is a diagrammatic representation showing derivation of coneshell venom peptide sequences. 5'RACE PCR using the primers AP1 + CHI-1B produce the 5' UTR and leader

15 peptide sequence which is then used to generate the PCR primers specific for χ-conotoxins. The 3' UTR using the primers CHI-1A + ANCHOR completed the derivation of the remaining mature peptide sequence and 3' UTR sequence.

20

25

- 16 -

EXAMPLES

Drugs

The drugs used in this and the following examples include: desipramine hydrochloride (Sigma);
5 indomethacin (Sigma); methoxamine hydrochloride (Sigma); (-)-noradrenaline bitartrate (Sigma); [^{3}H]-l-noradrenaline (specific activity 2200 Ci/mM; New England Nuclear, Boston, MA, U.S.A.); tetrodotoxin (Sigma); yohimbine hydrochloride (Sigma).

Statistical Analysis

10 Data for the examples below are expressed as the mean and standard error of 4-6 experiments. Sigmoidal curve-fitting for the calculation of EC_{50} values was performed by non-linear regression using the software package Igor Pro (WaveMetrics). Differences between means were assessed by Student's *t* test (two-tailed) using the software package Prism (GraphPad). Values of $P < 0.05$ were taken to indicate statistically significant differences.

15

Example 1

Rat vas deferens preparation

20 Male Wistar rats (250-350 g) were killed by a blow to the head and the vasa deferentia were removed. Each tissue was divided into bisected epididymal and prostatic portions. The tissue segments were mounted in 5 mL organ baths under a tension of 0.5 g. The bathing solution had the following composition (mM): NaCl, 119; KCl, 4.7; MgSO$_4$, 1.17; KH$_2$PO$_4$, 1.18; NaHCO$_3$, 25.0; glucose, 5.5; CaCl$_2$, 2.5; EDTA, 0.026; was maintained at 37°C and bubbled with 5% v/v
25 CO$_2$ in O$_2$. The preparations were equilibrated for at least 45 min prior to the commencement of experimentation. Contractions were registered by means of an isometric force transducer (Narco Bio-System F-60) and were recorded on a Power Macintosh computer using Chart version 3.5.4/s software and a MacLab/8s data acquisition system (ADInstruments) at a sampling frequency of 200 Hz.
30

The bisected prostatic segments were used to examine the effect of χ-MrIA on the electrically evoked contraction of the smooth muscle mediated by sympathetic neurotransmission. The tissue preparations were straddled with platinum stimulating electrodes. Electrical field stimulation (EFS) was made at 3 min intervals using a single 55 V pulse of 1 ms duration

5 generated by a Grass S44 Stimulator. The contractions could be blocked by tetrodotoxin (0.1 μM), indicating they were neurally mediated. Increasing concentrations of the peptide were added to the organ bath cumulatively in half log unit increments. Each dose was applied once the effect of the previous dose on the response to electrical stimulation had attained a steady level.

10

Effect of χ-MrIA on sympathetic neurotransmission

The bisected portions of the prostatic rat vas deferens responded to field stimulation with a biphasic contraction. In this preparation, the two components of the biphasic contraction were well separated temporally. The first part of the response was the dominant one, and reached a

15 maximum level approximately 200 ms after delivery of the electrical pulse. The second phase of the contraction peaked approximately 500 ms after stimulation. Our attempts to identify the pharmacological activity of χ-MrIA began with an investigation of its effects in the field stimulated rat vas deferens. The effect of χ-MrIA on the response of the preparation to field stimulation is shown in Figure 1. The conotoxin (30 nM-3 μM) acted to increase the second

20 phase of the contraction. This effect was found to be concentration dependent. By subtracting the control response from traces obtained in the presence of χ-MrIA , the specific enhancement by the peptide of only the second component of the contraction becomes apparent. A concentration-response curve for χ-MrIA acting to specifically potentiate the second component can also be constructed.

25

The action of χ-MrIA on the electrically evoked response was highly specific, enhancing only the second component of the biphasic response. This late phase of the contraction is recognised to be mediated by noradrenaline, and is selectively inhibited by prazosin and other α_1-

adrenoceptor antagonists. The first phase of the contraction, which is due to the activation of postjunctional P_{2x}-purinoceptors by released ATP, was not similarly enhanced. The magnitude of the noradrenergic component of the contraction is modulated by the amount of noradrenaline released by sympathetic nerve firing, the density of post-junctional α_1-adrenoceptors and their

5 coupling to effector systems, and the rate at which noradrenaline is cleared from the synapse.

Antagonism at presynaptic α_2-adrenoceptors is well recognised to enhance the electrically evoked release of noradrenaline from sympathetic nerves by blocking the activation of a negative feedback system by released noradrenaline. However, α_2-adrenoceptor antagonism can not be

10 the mechanism of action of χ-MrIA. Unlike χ-MrIA, α_2-adrenoceptor antagonists such as yohimbine and idazoxan act to enhance equally the purinergic and noradrenergic components of the contraction of the rat vas deferens. Furthermore, the response to a single pulse, as opposed to a train of stimuli, is not subject to regulation by this negative feedback mechanism since there would be no agonist present at these receptor sites at the time of stimulation.

15 Accordingly yohimbine (1 μM) has no effect on the evoked responses in this assay.

Example 2

Preparation to examine effect of χ-MrIA on responses to α_1-adrenoceptor agonists

20 The rat vas deferens was used as described above, except that the bisected epididymal segments were not electrically stimulated. These preparations were instead used to establish concentration response curves to noradrenaline and methoxamine in the absence and presence of χ-MrIA. In this tissue, noradrenaline and methoxamine cause contraction of the smooth muscle via activation of postjunctional α_1-adrenoceptors. χ-MrIA at a concentration of either 1 μM or 3

25 μM was applied to the organ bath and allowed to equilibrate with the tissue for 20 min prior to cumulative additions of noradrenaline or methoxamine. A single concentration response curve was determined per preparation, with contralateral tissue segments to which χ-MrIA was not applied serving as controls.

Effect of χ-MrIA on responses to α_1-adrenoceptor agonists

It was possible to determine whether the action of χ-MrIA occurs upstream or downstream of neurotransmitter release by examining the effect of the peptide on the response to exogenously

5 applied noradrenaline. Since χ-MrIA enhanced the potency of bath-applied noradrenaline, we can conclude that the conotoxin acts by potentiating the response to noradrenaline, rather than by promoting its release from neuronal stores. This potentiation could occur as a consequence of increased α_1-adrenoceptor responsiveness or impaired termination of the action of noradrenaline. The α_1-adrenoceptor agonist methoxamine was used to ascertain which of these

10 was the mechanism of action of χ-MrIA. This α_1-adrenoceptor agonist differs from noradrenaline in that it is not a substrate for the neuronal noradrenaline transporter. This transporter functions to eliminate noradrenaline and other catecholamines from the synapse by uptake into the nerve terminals, and represents the major mechanism for terminating the action of noradrenaline at the adrenoceptors of sympathetically innervated tissues. Because

15 methoxamine is not subject to removal by this mechanism, inhibition of the transporter does not enhance the potency of its actions.

The effect of χ-MrIA on the responses of the bisected segments of the rat epididymal vas deferens to two α_1-adrenoceptor agonists was investigated. Log concentration-response curves to noradrenaline in the absence and presence of χ-MrIA are shown in Figure 2a. At a

20 to noradrenaline in the absence and presence of χ-MrIA are shown in Figure 2a. At a concentration of 1 μM, χ-MrIA acted to increase the sensitivity of the tissue to noradrenaline, shifting the concentration response curve to the left. The degree of potentiation observed was larger in experiments with 3 μM χ-MrIA. Neither concentration of the conotoxin altered the maximum response of the tissue to noradrenaline. χ-MrIA at a concentration of 3 μM had no

25 effect on the concentration response curve to methoxamine (Figure 2b). The observation that χ-MrIA does not potentiate the action of methoxamine, in contrast to its effect on responsiveness to noradrenaline, is consistent with χ-MrIA being an inhibitor of the neuronal noradrenaline transporter.

The lack of effect of χ-MrIA on the concentration response curve to methoxamine also demonstrates that χ-MrIA has no effect at α_1-adrenoceptors, which would be evident as a parallel shift of the curve to the right. This distinguishes χ-MrIA from some other inhibitors

5 of noradrenaline transport, particularly those used as antidepressants. The therapeutic target of many of the antidepressant drugs is the neuronal noradrenaline transporter. However, many of these compounds, especially the tricyclic antidepressants, and to a lesser extent some newer drugs which are structurally unrelated to the conventional tricyclics, are recognised to act at other sites such as α_1-adrenoceptors and muscarinic ACh receptors.

10

Example 3

Guinea-pig ileum

Male guinea-pigs (285-425 g) starved overnight were killed by a blow to the head and

15 exsanguinated. Segments of the ileum of approximately 1.5 cm length were removed and the luminal contents cleared by gentle washing using a syringe filled with bathing solution. The preparations were placed in 5 mL organ baths containing bathing solution of the following composition (mM): NaCl, 136.9; KCl, 2.68; $CaCl_2$, 1.84; $MgCl_2$, 1.03; glucose, 5.55; $NaHCO_3$, 11.9; and KH_2PO_4, 0.45; warmed to 37°C and bubbled with 5% v/v CO_2 in O_2. Indomethacin (10

20 μM) was included in the bathing solution to produce a stable baseline. The tissues were placed under a tension of 1.0 g and allowed to equilibrate for 40 min prior to the commencement of experimentation. Doses of nicotine (4 μM) were then applied at 15 min intervals until the responses were observed to be consistent. χ-MrIA (3 μM) was then added, 25 min after which another dose of nicotine was applied. The responses to nicotine were measured isometrically and

25 digitised at a sampling rate of 10 Hz.

Effect of χ-MrIA on responses to nicotine in the guinea-pig ileum

χ-MrIA (3 μM) had no significant effect on the responses of ileal segments to nicotine. In the absence of the conotoxin, the mean response was 3.83 ± 0.76 g, compared to 4.07 ± 0.80 g when χ-MrIA was present (p > 0.1; paired t-test; n=4). The α-conotoxins block nicotinic ACh receptors of either the neuronal or muscle subtype. It was demonstrated that χ-MrIA does not target neuronal nicotinic ACh receptors using isolated segments of guinea-pig ileum. Because of the dependence of the contractile response on muscarinic receptor activation, it would be expected that the response of the guinea-pig ileum to nicotine would be attenuated in the presence of χ-MrIA if the conotoxin also acted as a muscarinic ACh receptor antagonist. In this preparation, the nicotine-induced release of ACh and various other neurotransmitters which activate postjunctional receptors was unaffected by χ-MrIA. Thus, and in contrast to many other transport inhibitors, χ-MrIA lacks anti-muscarinic activity.

Example 4

Mouse phrenic nerve-hemidiaphragm preparation

Male Quackenbush mice (20-30 g) were killed by cervical dislocation. Left and right hemidiaphragms were removed with the phrenic nerves attached. The base of each hemidiaphragm was positioned between two parallel platinum stimulating electrodes and the phrenic nerve was placed through two small platinum loops for field stimulation. The preparation was incubated at 37°C in à 5 mL organ bath bubbled with 5% v/v CO_2 in O_2. The composition of the bathing solution was (mM): NaCl, 135.0; KCl, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; glucose, 11.0; $NaHCO_3$, 15.0; and KH_2PO_4, 1.0. The tissues were placed under 1.0 g resting tension. After allowing at least 30 min for equilibration, alternating direct and indirect stimulation was made at 10 s intervals using a 30 V pulse of 2 ms duration to the muscle, and a 3 V pulse of 0.2 ms duration to the nerve, respectively. The effect of a single dose of χ-MrIA at a concentration of 3 μM on the directly and indirectly elicited contractions was examined. Responses were digitised and recorded as described for the vas deferens preparations.

Effect of χ-MrIA on responses to electrical stimulation of the mouse phrenic nerve-hemidiaphragm

Contractions elicited by field stimulation of the phrenic nerve, or by direct muscle stimulation, were unaffected by 3 µM χ-MrIA (n=4). χ-MrIA does not block the muscle nicotinic ACh receptors

5   in the mouse phrenic-nerve hemidiaphragm preparation.   The lack of activity of χ-MrIA at skeletal muscle or motor nerve distinguishes it from the majority of conotoxin peptides characterised to date which are paralytic toxins and hence have a clear role in prey capture.

Example 5

10

Cellular uptake of [^{3}H]-noradrenaline

Chinese hamster ovary (CHO) cells were grown in 24 well plates (Falcon) in 10% v/v fetal calf serum.   On reaching 60-70% confluence, the cells were transiently transfected (Lipofectamine, Gibco) with an expression vector (pcDNA3, Invitrogen) incorporating the full length cDNA for

15   the human neuronal noradrenaline transporter (Pacholczyk *et al.*, (1991) Nature, **350**, 350-4).   A cDNA clone of the neuronal noradrenaline transporter was used (Vollum Institute, Portland, OR, USA).   Cellular uptake studies were performed 36 hours after transfection.   The CHO cells were initially washed with transport buffer containing (mM): NaCl, 157; KCl, 2.7; NaH$_2$PO$_4$, 11.8; MgCl$_2$, 1.0 and CaCl$_2$, 0.1; and of pH 7.4.   The cells were then incubated with transport buffer

20   containing 50 nM [^{3}H]-noradrenaline (supplemented with unlabelled noradrenaline as required) and 100 µM ascorbic acid. χ-MrIA (0.1 nM-1 µM) or desipramine (10 µM) were also included as appropriate.   After 20 minutes at room temperature, the cells were rapidly washed with ice-cold phosphate buffered saline and then lysed in 0.1% v/v Triton-X.   The cell lysates were taken for liquid scintillation counting to determine their level of radioactivity.   Additionally, an aliquot

25   of the cell lysate was used to measure protein concentration (BioRad DC protein assay).   The specific uptake of [^{3}H]-noradrenaline by the noradrenaline transporter was defined as the component sensitive to desipramine (10 µM).

Effect of χ-MrIA on cellular accumulation of [³H]-noradrenaline

The accumulation of noradrenaline into CHO cells expressing the human neuronal noradrenaline transporter was reduced to less than 0.5% of the control amount by desipramine (10 μM), demonstrating that the accumulation was due to specific uptake via the cloned transporter. The

5 noradrenaline transporter was confirmed as the target of the conotoxin in cellular uptake studies. χ-MrIA (0.1 nM-1 μM) inhibited the accumulation of radiolabelled noradrenaline in a concentration-dependent manner (Figure 3), with a log IC_{50} value of -8.17 ± 0.0275 (n = 4). The accumulation of noradrenaline into the transfected CHO cells was demonstrated to be almost entirely due to specific uptake by the cloned transporter. The concentration of χ-MrIA

10 required to inhibit the accumulation by 50% was found to be approximately 7 nM. This concentration is approximately one order of magnitude lower than that needed for desipramine to produce the same effect.

Cocaine and χ-MrIA are both naturally occurring compounds, however, they are quite

15 dissimilar. Cocaine is an alkaloid extracted from the leaves of the coca plant, whereas χ-MrIA is a peptide directly encoded by an animal gene. In addition to its effect at the uptake₁ transporter, cocaine is known to possess potent local anaesthetic properties. This is due to blockade of both sodium and potassium channels. No evidence was found for local anaesthetic activity of χ-MrIA in any of the assays. It was found that χ-MrIA had neither contractile nor

20 relaxant effects on the tone of the vas deferens by itself. Similar studies revealed that χ-conotoxin does not inhibit the dopamine transporter.

Example 6

Tritiated mazindol binding to the noradrenaline transporter was measured in cells expressing the

25 transporter protein (see Example 5). The influence of χ-MrIA from 10^{-6} to 10^{9} on tritiated mazindol binding was measured. χ-MrIA had no effect on tritiated maizindol binding, indicating that it acts allosterically, at a site distinct from traditional noradrenaline transport inhibitors, such as desipramine, mazindol and cocaine.

- 24 -

Example 7

Derivation of gene sequence for the χ-conotoxin peptides

The complete gene sequence for the χ-MrIA was isolated using a combined 5' RACE (<u>R</u>andom

5 <u>A</u>mplification of <u>c</u>DNA <u>E</u>nds) and 3' RACE strategy coupled with cloning and DNA

sequencing.

5' RACE

The oligonucleotide primer CH1-1B were designed from the mature peptide sequence. The

10 relationship of the oligonucleotides to the peptide is as follows, together with the oligonucleotide

sequence:

| χ-MrIA | - | NGVCCG<u>YKLCHPC</u> | SEQ ID NO. 3 |

15   CH1-1B

CH1-1B 5' -   CANGGRTGRCANARYTTRTA - 3'   SEQ ID NO. 4

AP1   5' -   CCATCCTAATACGACTCACTATAGGGC -3'   SEQ ID NO. 5

20

(where N=A/C/G/T, R=A/G, Y=C/T,)

Polymerase Chain Reaction (PCR) was carried out using the oligonucleotide CH1-1B in

25 combination with the AP1 oligonucleotide on cDNA templates derived from the mRNA isolated

from coneshell venom ducts. The PCR products, which represent the 5' region of the MrIA

gene were isolated, purified, cloned into bacterial vectors and sequenced. Gene sequence for

MrIA was obtained from *C. marmoreus* (Fig. 4).

3' RACE

The DNA sequence for the 5'-regions of the gene was used to design oligonucleotides that were capable of detecting the MrIA sequence, and sequences from other closely related peptides. The positioning of the oligonucleotides relative to the gene sequence is shown in Figure 4. The oligonucleotide CH1-1A was used in PCR in conjunction with the ANCHOR oligonucleotide to produce DNA fragments corresponding to the leader peptide, mature peptide and 3' untranslated (3'UTR) regions of the gene. PCR of venom duct cDNA templates from *C. marmoreus* produced DNA fragments corresponding to the MrIA peptide.

The DNA sequences for the oligonucleotides are:

CH1-1A   5' -   ACAGGCAGAATGCGCTGTCTCCC-3'   SEQ ID NO. 6

ANCHOR 5' -   AACTGGAAGAATTCGCGGCCGCAGGAAT-3'   SEQ ID NO. 7

Complete sequence for χ-MrIA

Gene sequence for χ-MrIA produced using 5' RACE and 3' RACE represent overlapping fragments of the gene. These fragments were combined, to produce a consensus sequence for the gene. The consensus sequence is the full cDNA for the gene, and includes 5' UTR, the leader peptide, the mature peptide and the 3' UTR.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the

steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

- 27 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5
    (i) APPLICANT: UNIVERSITY OF QUEENSLAND
    (ii) TITLE OF INVENTION: NOVEL PEPTIDES - I

    (iii) NUMBER OF SEQUENCES: 7

10
    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: DAVIES COLLISON CAVE
        (B) STREET: 1 LITTLE COLLINS STREET
        (C) CITY: MELBOURNE
15
        (D) STATE: VICTORIA
        (E) COUNTRY: AUSTRALIA
        (F) ZIP: 3000

    (v) COMPUTER READABLE FORM:
20
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25
    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: AU PROVISIONAL
        (B) FILING DATE: 2-OCT-1998
        (C) CLASSIFICATION:

30
   (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: MICHAEL J CAINE
        (C) REFERENCE/DOCKET NUMBER: MJC/RR

    (ix) TELECOMMUNICATION INFORMATION:
35
        (A) TELEPHONE: +61 3 9254 2777
        (B) TELEFAX: +61 3 9254 2770
        (C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
5   (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide
10
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Gly Val Cys Cys Gly Tyr Lys Leu Cys His Hyp Cys
15   5   10

    (2) INFORMATION FOR SEQ ID NO:2:
20
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
30

Val Gly Val Cys Cys Gly Tyr Lys Leu Cys His Hyp Cys
              5   10

35

    (2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
40   (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

45   (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

50 Asn Gly Val Cys Cys Gly Tyr Lys Leu Cys His Pro Cys
              5   10

55

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
5   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA
10

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15
CANGGRTGRC ANARYTTRTA   20

20
(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
25   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA
30

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35
CCATCCTAAT ACGACTCACT ATAGGGC   27

40 (2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
45   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA
50

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

55 ACAGGCAGAA TGCGCTGTCT CCC   23

    (2) INFORMATION FOR SEQ ID NO:7:

60   (i) SEQUENCE CHARACTERISTICS:

- 30 -

```
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
5
    (ii) MOLECULE TYPE: DNA


10    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

   AACTGGAAGA ATTCGCGGCC GCAGGAAT                                    28
15
```

20 DATED this 2nd day of October, 1998

The University of Queensland

By DAVIES COLLISON CAVE
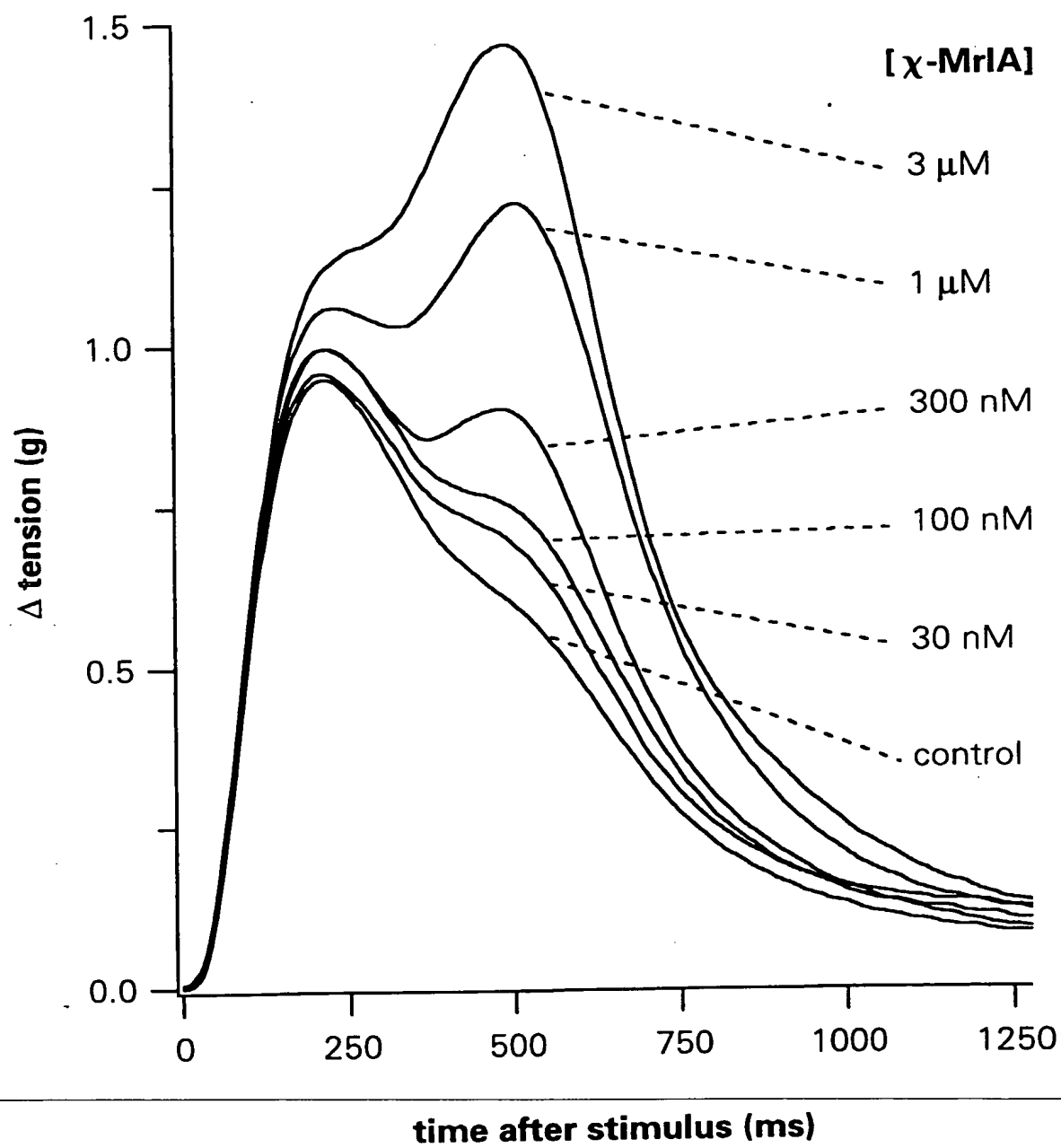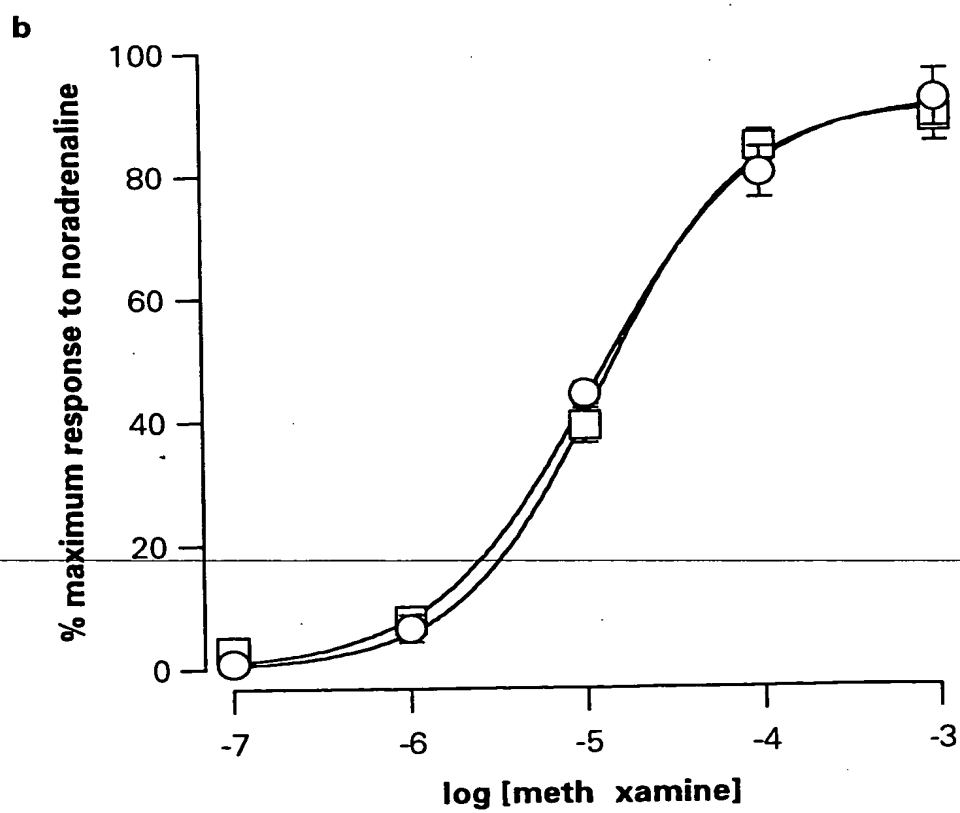
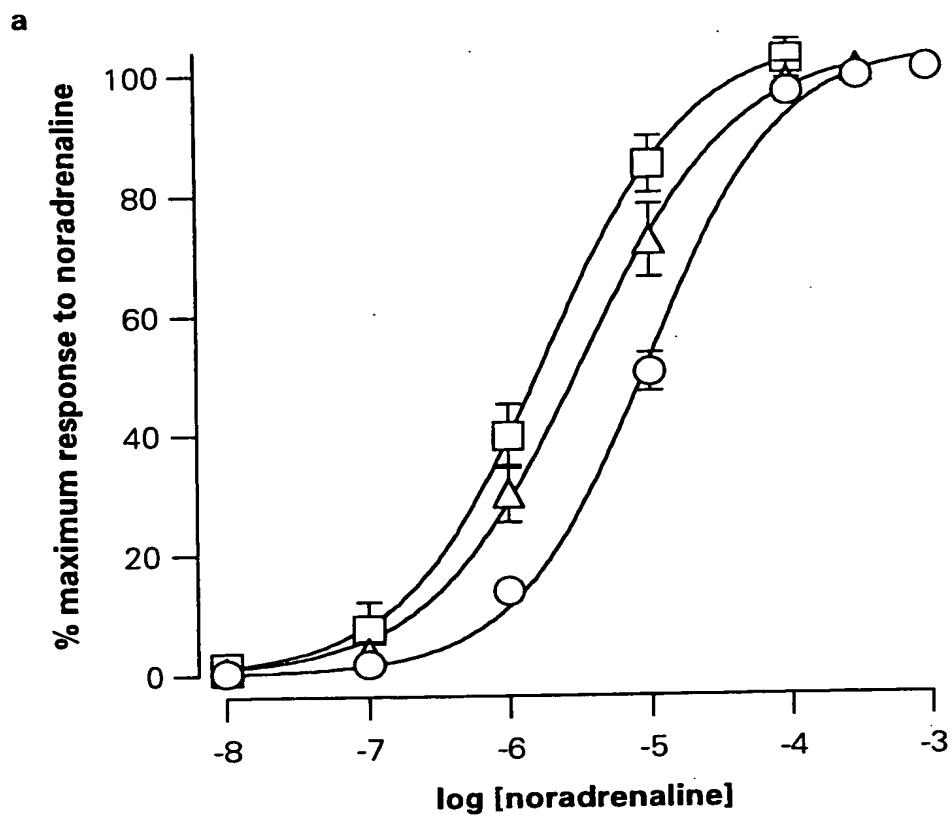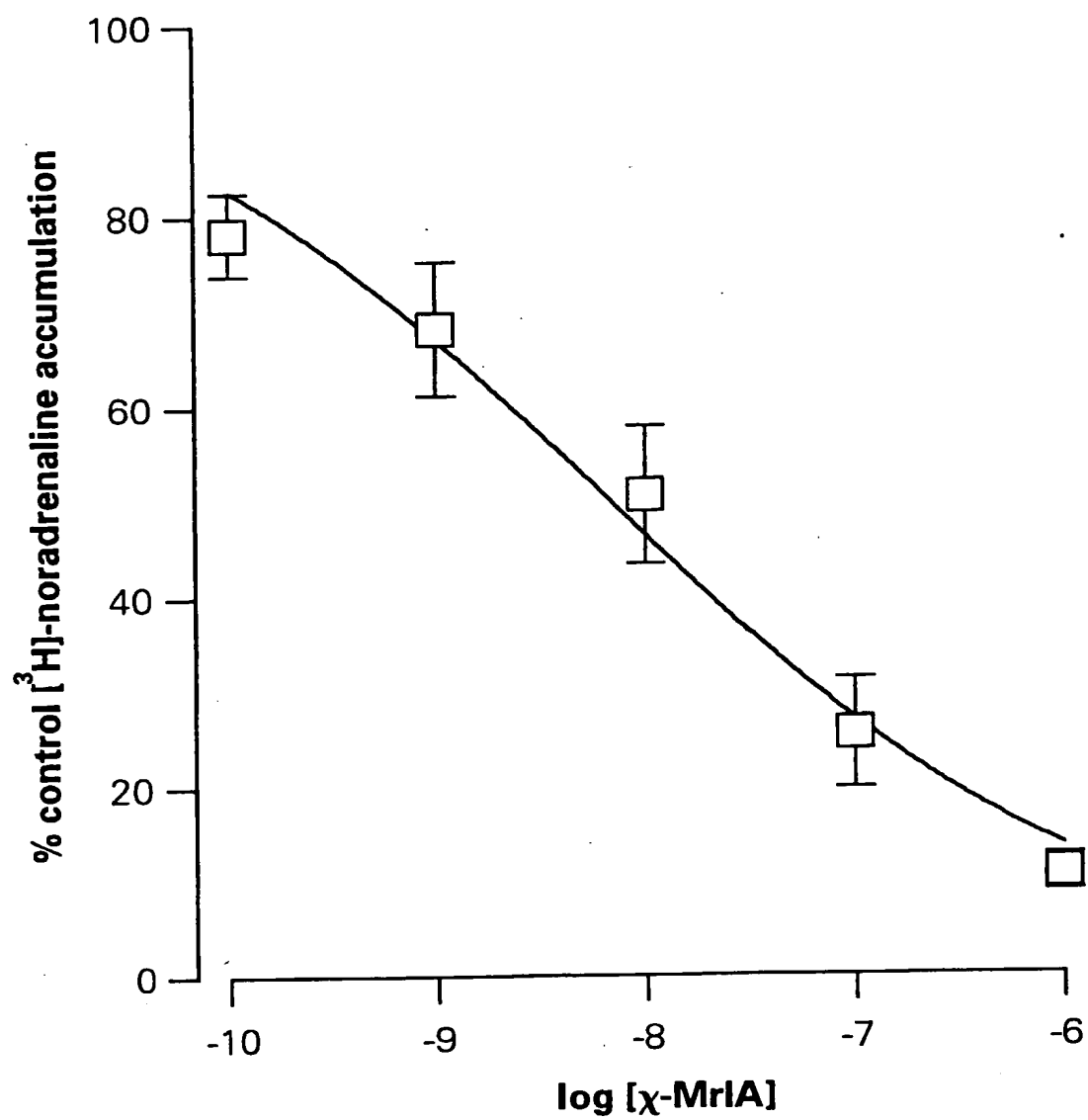25 Patent Attorneys for the Applicant

FIGURE 1

FIGURE 2

a

b

FIGURE 3

FIGURE 4